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Three-dimensional matrix suppresses E2F-controlled gene expression in glomerular mesangial cells

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Three-dimensional matrix suppresses E2F-controlled gene expression in glomerular mesangial cells.

Background. Extracellular matrix (ECM) regulates mitogenesis of glomerular mesangial cells. Currently, however, the molecular mechanisms that mediate the control of cell growth by ECM are not fully elucidated.

Methods. The effects of structurally distinct forms of type I collagen matrix on mesangial cell proliferation and cell cycle distribution were examined. Expressions of the cell cycle-regulatory transcription factor E2F and retinoblastoma susceptibility gene family proteins were also investigated.

Results. Mesangial cells cultured on monomeric collagen matrix showed a substantial growth response to serum. In contrast, mesangial cells cultured on polymerized collagen matrix exhibited arrest of the cell cycle in the G0/G1 phase. The induction of the quiescent phenotype was correlated with down-regulation of E2F-1, the prototypal transcription factor that controls cell cycle progression. The suppression of E2F-1 was associated with (*1*) dephosphorylation of retinoblastoma susceptibility gene proteins, pRB and p130, and (*2*) accumulation of E2F-pRB and E2F-p130 DNA binding complexes that bind to the E2F consensus sequence located in the E2F-1 promoter. Other E2F regulatory genes, including c-*myc*, cyclin A, and *cdc 2*, were also down-regulated in mesangial cells cultured on polymerized collagen matrix.

Conclusion. These results suggest that a three-dimensional collagen induces cell cycle arrest via suppression of E2F-controlled gene expression in mesangial cells. Dephosphorylation of pRB and p130 and subsequent generation of transrepressor complexes, E2F-pRB and E2F-p130, may be involved in this process.

Cell proliferation is an important biologic event involved in a wide range of pathophysiological processes. Mitogenesis is tightly controlled by many soluble regulators, including cytokines, growth factors, autacoids, and hormones [1, 2]. However, in many cases, cellular responses to soluble factors are affected by other environmental factors. Accumulating data have shown that extracellular matrix (ECM) modulates cell proliferation via specific cell membrane receptors of the integrin family [3]. Ligation of integrins can transduce certain signaling pathways [4, 5] and can regulate transcription factors [6, 7], leading to altered gene expression. However, the molecular mechanisms that mediate control of cell growth by ECMs are not fully elucidated.

The antimitotic action of three-dimensional ECMs on mesangial cells is of particular interest. Mesangial cells proliferate actively when cultured on plastic substrata or plates coated with monomeric collagens [8]. In contrast, mesangial cells show repressed mitotic activity when cultured on or within polymerized three-dimensional ECMs. These include interstitial-type collagen matrix, basement membrane-type matrix, or matrices produced by mesangial cells during prolonged cultures [9, 10]. In the normal glomerulus, mesangial cells reside in the three-dimensional ECM, namely, mesangial matrix, which contains collagen types IV and V, laminin, fibronectin, and proteoglycans, and have a low proliferative activity [11]. In pathologic situations, proliferation of mesangial cells occurs in parallel with destruction and/or qualitative alterations of the mesangial matrix, and collagen types I and III are often detected in the mesangium [12–15]. These observations suggest a crucial role of three-dimensional spatial organization as well as ECM composition in the regulation of mesangial cell proliferation.

A recent advance in cell cycle biology has demonstrated that many genes required for cell cycle progression are governed by the E2F family of molecules [16]. For example, E2F plays a crucial role in the transcriptional regulation of c-*myc*, cyclin A, *cdc 2*, and E2F-1 [17–20]. The activity of E2F is regulated by molecular interaction with other proteins. Retinoblastoma susceptible gene proteins, pRB, p107, and p130, bind to E2F

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and inhibit its function [16]. Recent studies have revealed that E2F is a target of some growth inhibitory factors such as interferons, transforming growth factor-β, and all-*trans* retinoic acid [21–23]. To explore molecular mechanisms involved in the antimitotic action of three-dimensional ECMs, the present study investigated whether and how structurally distinct forms of collagen matrix modulate the activity of the cell cycle-regulatory machinery, especially the E2F-dependent pathway.

METHODS

Cells

Human mesangial cells were purchased from Clonetics Corporation (Walkersville, MD, USA). Cells (5th through 8th passages) were maintained in MCDB-131 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 100 U/mL penicillin, and 100 μg/mL of streptomycin (GIBCO BRL, Gaithersburg, MD, USA).

Collagen matrices

Polymerized collagen was prepared by neutralizing the type I collagen solution (Cellmatrix™ type I-P; Nitta Gelatin, Osaka, Japan). In brief, collagen solution was mixed on ice with (*1*) 1/10 volume of 10 × concentrated MCDB-131 without $NaHCO_3$, and (*2*) 1/10 volume of 2.2% $NaHCO_3$ and 0.05 N NaOH with 200 mmol/L HEPES. The mixture was poured into 100 mm culture plates (0.08 mL/cm^2) and incubated at 37°C for one hour. To prepare monomeric collagen, culture plates were incubated with 0.1 mg/mL of type I collagen solution in 1 mmol/L HCl for one hour at room temperature and then washed with MCDB 131 medium.

Proliferation and cell cycle analysis

Cells (4×10^5) were seeded in 100 mm dishes coated with either monomeric or polymerized collagen in the presence of 10% FBS, and cell count and cell cycle analysis were performed at the indicated time points. To harvest cells on polymerized collagen, collagen gels were digested with 2 mg/mL collagenase (collagenase S-1; Nitta Gelatin) until cells were dispersed completely. For monomeric collagen, cells were washed twice with phosphate buffered saline (PBS) and digested with 0.01% trypsin, containing 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA). The cell count was assessed by trypan blue staining. Cells were mixed with same volume of 0.4% trypan blue solution, and only viable cells were counted by light microscopy. Cell cycle analysis was performed as follows: Sixty minutes prior to harvest, cells were labeled with 10 μmol/L bromodeoxy uridine (BrdU), and single-cell suspensions were prepared as described previously in this article. Cells were fixed in 70% ethanol and denatured by 2 N HCl/0.5% Triton X for 30 minutes at room temperature, followed by neutralization with $Na_2B_4O_7$ (pH 8.5). The cells were subjected to dual color staining with anti-BrdU antibody conjugated with FITC (Becton Dickinson, San Jose, CA, USA) and 5 μg/mL propidium iodide. Two × 10^4 cells of each samples were evaluated, and all analyses were carried out using the FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson). Assays were performed in quadruplicate.

Reverse-transcription polymerase chain reaction analysis

Total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) kit according to the manufacture's protocol. The first-strand cDNA was synthesized using M-MLV-reverse transcriptase (GIBCO BRL). Six micrograms of total RNA were transcribed using a random hexamer primer (Pharmacia Biotech, Uppsala, Sweden) in a reaction mixture with a total volume of 30 μL. The PCR was done using 1.5 μL of first-strand cDNA and a thermal cycler (Gene AMP PCR system 2400; Perkin Elmer, Norwalk, CT, USA). Each cycle of PCR consisted of one minutes of denaturation at 94°C, one minute of annealing at 60°C, and two minutes of extension at 70°C. Each reverse-transcription polymerase chain reaction (RT-PCR) product (5 μL) was electrophoresed on 2% agalose gels. Then the gels were stained with ethidium bromide and photographed using an ultraviolet transilluminator. Control experiments were performed to determine the range of PCR cycles over which amplification efficiency remained constant and to confirm that the amount of amplified PCR product was directly proportional to the amount of applied RNA (data not shown). The following oligonucleotides were used as primers:

E2F-1 sense primer, GACTCCTCGCAGATCGTCATC
E2F-1 antisense primer,
ACGAAGGTCCTGACACGTCAC
E2F-4 sense primer,
ACACCCGGCCACTGCAGTCTTCT
E2F-4 antisense primer,
TCCAGGTTGTAGATATAATCGTGGT
pRB sense primer, GGACCGAGAAGGACCAACTGA
pRB antisense primer,
CTCAGACAGAAGGCGTTCACA
p107 sense primer, CAGCACAGGCTAATGTGGAGT
p107 antisense primer,
TGATGCAACAGGAGTAATGACTG
p130 sense primer, CAAGATGCGTTCCACAGATCT
p130 antisense primer,
CAAGTCCTCCAGTATCAGCACGA
c-Myc sense primer, AAGACTCCAGCGCCTTCTCTC
c-Myc antisense primer,
GTTTTCCAACTCCGGGATCTG

cyclin A sense primer,
CCAGACTACCATGAGGATATT
cyclin A antisense primer,
GTTTGCAGGCTGCTGATGCAG
cdc 2 sense primer, TCTATCCCTCCTGGTCAGTAC
cdc 2 antisense primer,
ATGGGATGCTAGGCTTCCTGG
β-actin sense primer, GTGGGGCGCCCCAGGCACCA
and β-actin antisense primer,
CTCCTTAATGTCACGCACGATTTC

Northern blotting

Total RNA (10 μg) was electrophoresed in a 1% agarose gel containing 6% formaldehyde, 20 mmol/L MOPS, 5 mmol/L sodium acetate, and 1 mmol/L EDTA, and was blotted onto the Hybond N^+ synthetic nylon membrane (Amersham Corp., Arlington Heights, IL, USA). The membrane was hybridized with each cDNA probe, which was labeled with [^{32}P] dCTP using the oligonucleotide random priming method. The following cDNAs were used in this study: a 1.4-kb EcoRI-Bam HI fragment of human E2F-1 cDNA (provided by Drs. William G. Kaelin, Jr., James A. DeCaprio, and David M. Livingston; Dana-Faber Cancer Institute, Boston, MA, USA) [24], and a 1.2 kb full-length fragment of human E2F-4 cDNA (generated by RT-PCR based on the published sequence [25]). Densitometric analysis was performed using NIH image 1.58 software.

Western blotting

Cells were washed with ice-cold TBS buffer (25 mmol/L Tris-HCl, pH 8.0, 120 mmol/L NaCl) and lyzed in EBC buffer (50 mmol/L Tris-HCl, pH 8.0, 120 mmol/L NaCl, 0.5% Nonidet P-40, 100 mmol/L sodium fluoride, 200 mmol/L sodium orthovanadate) containing protease inhibitors (aprotinin 10 μg/mL, phenylmethylsulfonyl fluoride 10 μg/mL, and leupeptin 10 μg/mL). An equal amount of protein (50 μg for E2F-1, p107, and p130; 120 μg for pRB) was separated on a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel. For detection of E2F-1, p107, and p130 protein, blots were preincubated in TBS buffer containing 5% nonfat dry milk and were incubated with 1 μg/mL of primary antibodies in TBS buffer containing 5% nonfat dry milk. For visualization, the enhanced chemiluminescence system (Amersham Corp.) was used according to the manufacturer's protocol. For the detection of pRB, the proteins were transferred onto nitrocellulose membrane. After blocking in TBS buffer containing 4% bovine serum albumin (fraction V; Sigma Chemical Co.), the membrane was incubated with 5 μg/mL of primary antibodies in TBS containing 0.05% Tween 20 and 4% bovine serum albumin. Then the membrane was probed with alkaline phosphatase-conjugated rabbit antimouse IgG (Promega, Madison, WI, USA) and developed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as described previously [26]. Primary antibodies used in this study were as follows: anti–E2F-1 (C20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-p107 (C18; Santa Cruz Biotechnology), anti-p130 (Transduction Laboratories, Lexington, KY, USA), and anti-pRB (PMG3-2459; Pharmingen, San Diego, CA, USA).

Gel retardation assay

Whole cell extract was prepared described previously in this article. Samples (2 to 10 μg) were incubated with approximately 0.5 ng (1×10^4 cpm) of ^{32}P-labeled DNA fragments containing the E2F binding site within the E2F-1 promoter (5′-ATTTAAGT*TTCGCGC*CCTTTC TCAA-3′; Santa Cruz Biotechnology) in the presence of 1 μg of poly dI-dC in a final volume of 25 μL. Incubations were carried out at room temperature for 30 minutes in 20 mmol/L HEPES, pH 7.9, 0.1% Nonidet P-40, 40 mmol/L KCl, 1 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 0.5 mmol/L dithiothreitol, and 10% glycerol. The protein-DNA complexes were resolved in 4% polyacrylamide gels (acrylamide:bisacrylamide ratio, 86:1 wt/wt) in 0.25 × Tris borate-EDTA buffer at 4°C. For antibody perturbation experiments, 1 μg of each antibody was added to the reaction mixture. Specific antibodies used in this study were as follows: anti-pRB (C36; Pharmingen), anti-p107 (C-18; Santa Cruz Biotechnology), and anti-p130 (Transduction Laboratories).

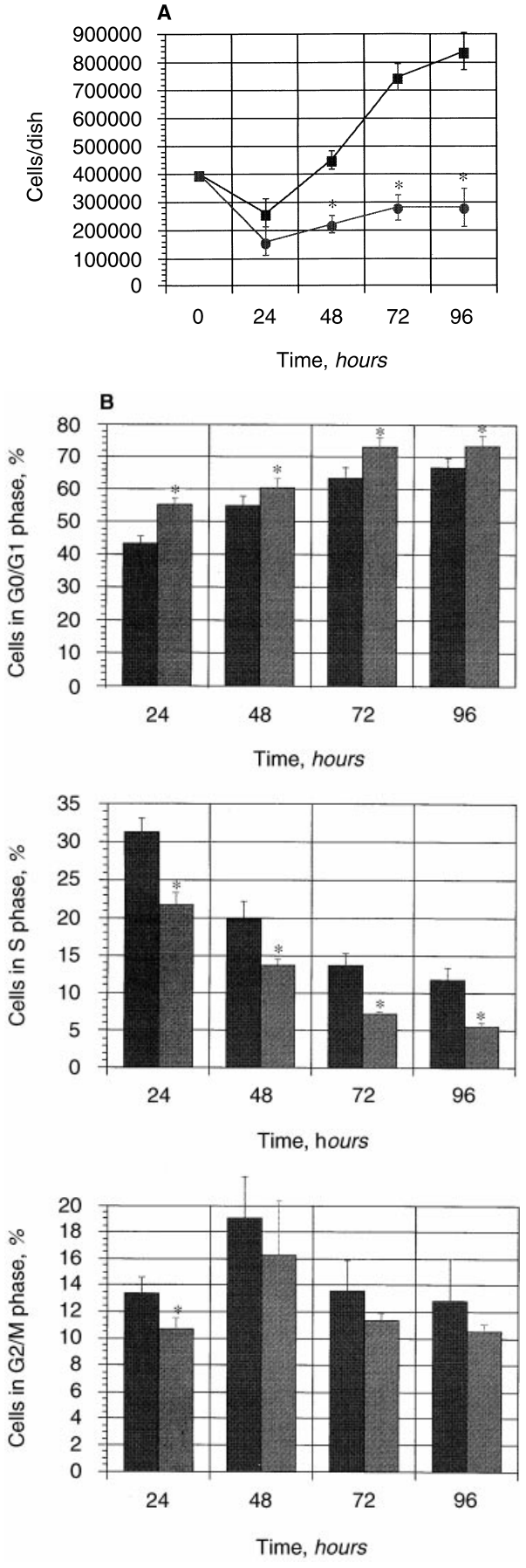
Statistical analysis

All experiments were repeated three or four times, and representative data are shown. Data are expressed as mean ± SD. Statistical analysis was performed using Student's *t*-test.

RESULTS

Effects of structurally distinct forms of collagen matrices on mesangial cell proliferation and cell cycle distribution

Mesangial cells were seeded at a density of 4×10^5 cells/100 mm dish precoated with either monomeric or polymerized collagen. Compared with the culture on monomeric collagen, proliferation of mesangial cells cultured on polymerized collagen was significantly inhibited. After 96 hours, the number of cells on monomeric collagen increased to 8.5×10^4 per dish, whereas the increase in cell number was not observed in the cells on polymerized collagen (Fig. 1A). The growth inhibitory effect of polymerized collagen was confirmed by cell cycle analysis. In the cells on monomeric collagen, the proportion of S phase was 31.3 ± 1.8% after 24 hours and 11.7 ± 1.7% after 96 hours (means ± SD). When cultured on polymerized collagen, the S phase fraction was 21.8 ± 1.7% after 24 hours and 5.6 ± 0.4% after 96



hours (Fig. 1B). Conversely, the proportion of cells in G0/G1 phase increased gradually, and after 96 hours, 73.4 ± 1.9% of the cells were in G0/G1 phase when cultured on polymerized collagen (vs. 66.9 ± 3.3% on monomeric collagen; Fig. 1B). The proportion of cells in G2/M phase did not change on polymerized collagen, although a transient increase was observed at 48 hours (Fig. 1B). These results suggest that polymerized collagen inhibited cell cycle progression of mesangial cells via intervention in G1-S boundary and subsequent induction of G0/G1 phase arrest.

Effects of collagen matrices on expression of E2F-1 and E2F-4, the major members of the E2F family of transcription factor

Polymerized collagen induced G0/G1 phase arrest in mesangial cells. To explore the molecular mechanisms involved, we examined whether polymerized collagen affects the level of E2F, the critical regulator of the cell cycle progression. Total RNA was extracted from mesangial cells grown on either monomeric or polymerized collagen matrix, and expression of E2F-1 and E2F-4 was examined by RT-PCR and Northern blot analyses. As shown in Figure 2A, the level of E2F-1 mRNA decreased within 24 hours after culturing the cells on polymerized collagen. In contrast, the level of E2F-4 mRNA was unaffected during the initial 72 hours, although a modest decrease was observed after 96 hours in the cells on polymerized collagen. These results were further confirmed by Northern blot analysis (Fig. 2B).

To examine whether the depressed level of E2F-1 mRNA is associated with down-regulation of E2F-1 protein, Western blot analysis was performed. Whole cell lysates were immunoprecipitated with an anti-E2F-1–specific antibody to detect the E2F-1 protein. Consistent with the reduction in mRNA levels, E2F-1 protein was down-regulated within 24 hours after culture on polymerized collagen matrix (Fig. 2C). A decrease in E2F-1 protein was not detected in the cells cultured on monomeric collagen.

Effects of collagen matrices on DNA binding activity of E2F

Because of marked reductions in E2F-1 expression in the cells cultured on polymerized collagen, we next

Fig. 1. Effects of structurally distinct forms of collagen matrices on mesangial cell proliferation. Cells were cultured at an initial density of 4×10^5 cells/100 mm dishes in the presence of 10% FBS on either monomeric or polymerized collagen for 96 hours. The cell number at indicated time points (*A*) and cell cycle distributions on each condition (*B*) are shown. Statistical significance was analyzed by comparing cells cultured on polymerized collagen with those cultured on monomeric collagen at each time point. Symbols in A are: (■) monomer; (●) polymer. Symbols in B are: (■) monomer; (▒) polymer. $*P < 0.05$ vs. monomeric collagen.

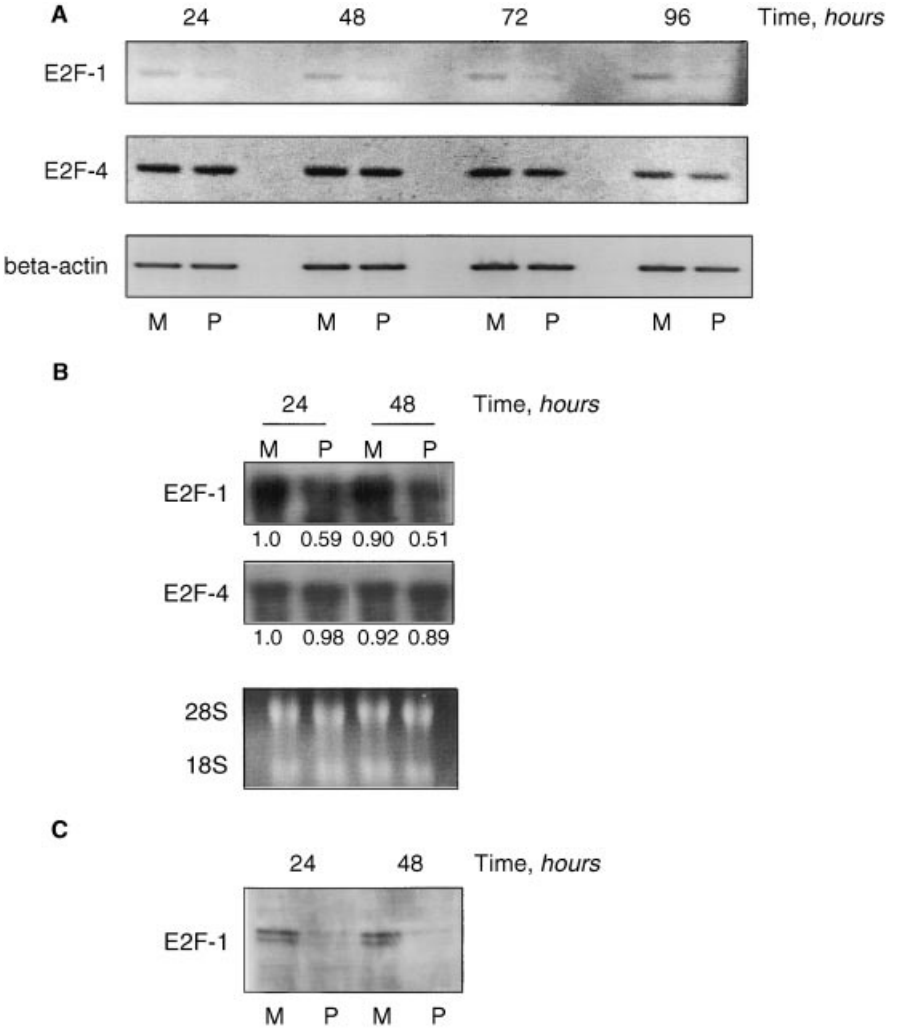


Fig. 2. Effects of collagen matrices on expression of E2F-1 and E2F-4, the major members of the E2F family of transcription factor. Cells were cultured in the presence of 10% FBS on either monomeric (M) or polymerized (P) collagen for 96 hours. (*A*) Total cellular RNAs were isolated at the indicated time points and subjected to RT-PCR analysis for E2F-1 and E2F-4 mRNA expression. The expression of β-actin gene is shown as an internal control. The number of PCR cycle was set in each gene to demonstrate that the amount of amplified PCR product was directly proportional to the amount of input RNA (23 cycle for E2F-1 and E2F-4, 19 cycle for β-actin). (*B*) Northern blot hybridization for E2F-1 and E2F-4 mRNA expression at 24 hours and 48 hours of culture on monomeric or polymerized collagen. Relative densitometric values were indicated. Ethidium bromide-stained ribosomal RNA is shown as a loading control. (*C*) Whole cell lysates were prepared at 24 and 48 hours of culture on either monomeric or polymerized collagen and were subjected to Western blot analysis using anti-E2F-1–specific monoclonal antibodies.

investigated the DNA binding ability of E2F to its consensus sequence within the E2F-1 promoter. Whole cell extracts were prepared from the cells cultured on either monomeric or polymerized collagen and subjected to gel retardation assays using an E2F consensus sequence. As shown in Figure 3A, three-band retarded mobilities (designated as A to C) were observed. In the cells on monomeric collagen, band C was predominant over the periods of the experiment. In contrast, in the cells on polymerized collagen, band A and B emerged, and the intensity of band C was decreased markedly following incubation for 48 hours. All of these three bands disappeared by the addition of 100 molar excess of unlabeled E2F oligonucleotide (Fig. 3B).

We performed antibody perturbation experiments to identify the components of each complex. Previous reports have demonstrated that RB family proteins (pRB, p107, and p130) were involved in the E2F protein complexes. To examine this possibility, specific antibodies against pRB, p107, and p130 were included in the reaction mixture containing whole cell extracts prepared from cells cultured on polymerized collagen for 72 hours. Gel retardation assay was carried out as described previously in this article. The anti-p130 antibody attenuated the intensity of band A, and the anti-pRB antibody diminished band B (Fig. 3C). The anti-p107 antibody did not obviously affect bands A and B. These results indicate that band A represents a complex containing E2F-p130, and band B is mainly composed of E2F-pRB. Since any specific antibodies against RB family proteins did not affect the intensity of band C (data not shown), this band represents free E2F that activates transcription of many growth promoting genes [27, 28].

Effects of collagen matrices on phosphorylation status of RB family proteins

Previous reports have shown that RB family proteins were activated via dephosphorylation [16, 29]. Since E2Fs formed complexes with pRB and p130 in mesangial cells on polymerized collagen, the phosphorylation state

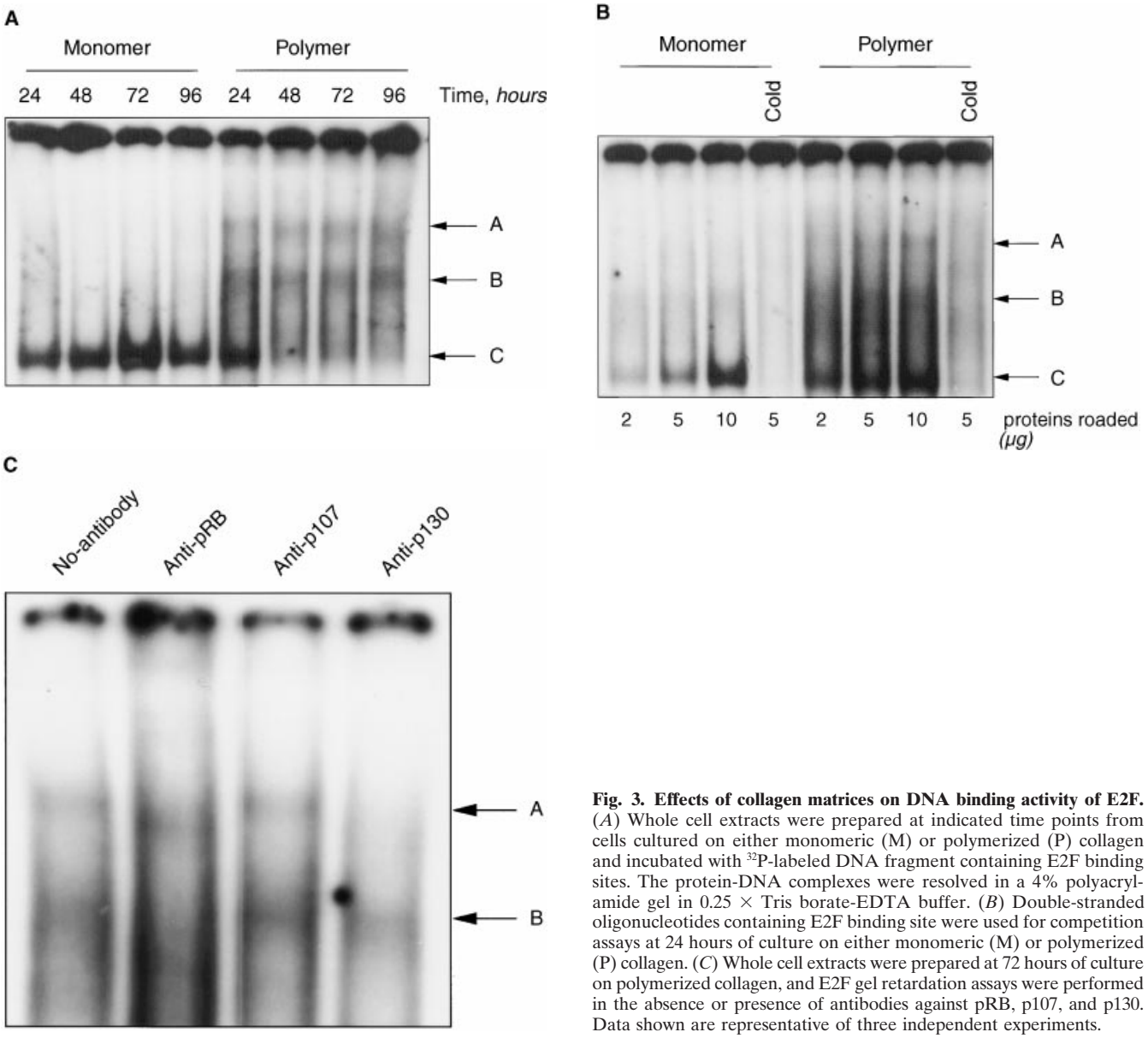


Fig. 3. Effects of collagen matrices on DNA binding activity of E2F. (*A*) Whole cell extracts were prepared at indicated time points from cells cultured on either monomeric (M) or polymerized (P) collagen and incubated with ^{32}P-labeled DNA fragment containing E2F binding sites. The protein-DNA complexes were resolved in a 4% polyacrylamide gel in 0.25 × Tris borate-EDTA buffer. (*B*) Double-stranded oligonucleotides containing E2F binding site were used for competition assays at 24 hours of culture on either monomeric (M) or polymerized (P) collagen. (*C*) Whole cell extracts were prepared at 72 hours of culture on polymerized collagen, and E2F gel retardation assays were performed in the absence or presence of antibodies against pRB, p107, and p130. Data shown are representative of three independent experiments.

of these RB family proteins was studied. Whole cell lysates were prepared from cells cultured on polymerized collagen matrix and subjected to immunoblot analysis. As shown in Figure 4A, polymerized collagen matrix induced a gradual decrease in the level of phosphorylated pRB. After 96 hours, almost all of the proteins were in the nonphosphorylated form. In contrast, on monomeric collagen, phosphorylated forms of pRB were present at the same time point. Similarly, most of phosphorylated p130 were shifted to the nonphosphorylated form within 48 hours of culture on polymerized collagen. In the cells on monomeric collagen, the phosphorylated form of p130 was retained even at 96 hours of culture.

Interestingly, p107, which was detectable in the cells cultured on monomeric collagen, disappeared in the cells on polymerized collagen (Fig. 4A, bottom). RT-PCR analysis revealed that the suppression occurred at the transcriptional level (Fig. 4B). On the other hand, mRNA levels of pRB and p130 were not altered (data not shown).

Effect of polymerized collagen on mRNA expression of the E2F-controlled genes

The c-*myc*, cyclin A, and *cdc 2* genes have E2F binding sites in its promoter regions [17–19], and their transcription is regulated by E2F. To further examine the activity of E2F binding sites in mesangial cells cultured on polymerized collagen matrix, we investigated the expression of E2F-controlled genes using RT-PCR analysis. As

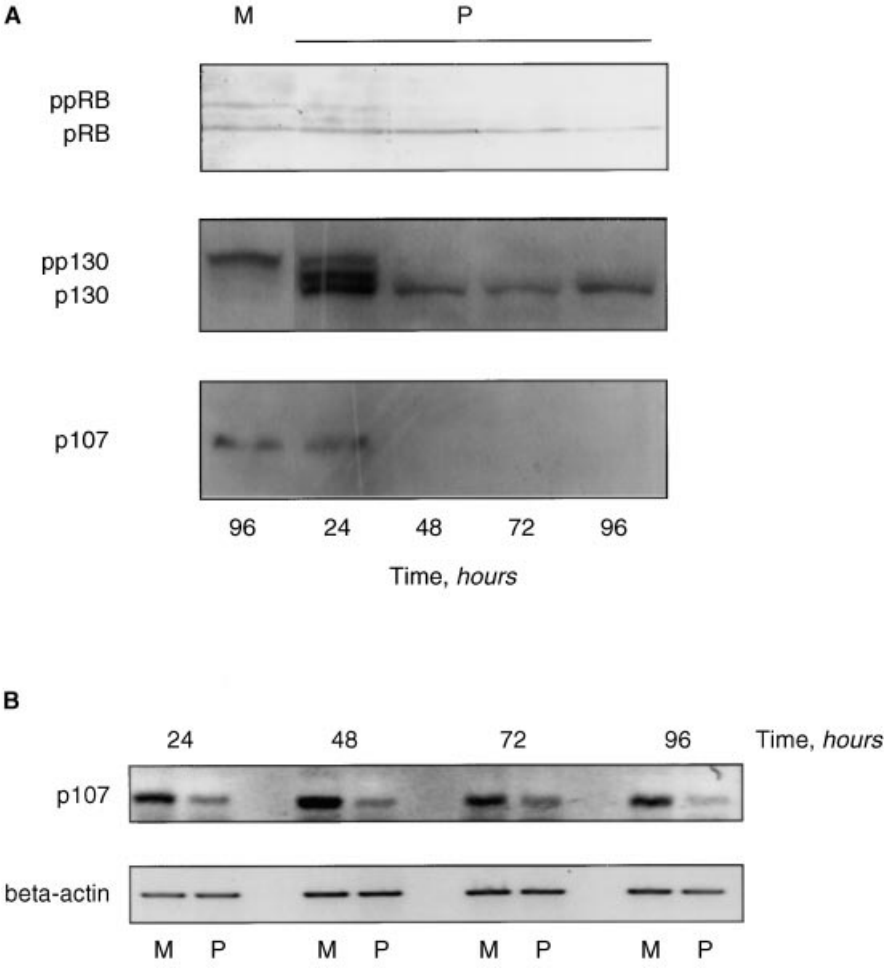


Fig. 4. Phosphorylation status of RB family proteins and mRNA expression of p107 on polymerized collagen matrix. (*A*) Whole cell extracts were prepared at indicated time points from cells cultured on either monomeric (M) or polymerized (P) collagen and were subjected to immunoblotting with antibodies against pRB, p130, and p107. The positions of nonphosphorylated RB and 130 protein (pRB and p130) and phosphorylated RB and p130 protein (ppRB and pp130) are indicated. (*B*) Total cellular RNA was isolated at the individual time point and subjected to RT-PCR analysis for p107 mRNA expression (25 cycle) as described in the legend of Figure 2. The expression of β-actin gene is shown as an internal control.

shown in Figure 5, polymerized collagen significantly inhibited the expression of c-*myc*, cyclin A, and *cdc 2*. Suppression of these growth-promoting genes may contribute to the antimitotic effect initiated by the polymerized collagen matrix.

DISCUSSION

Adhesion is a primary step required for proliferation of normal diploid cells [30–32]. However, mesangial cells cultured on or within three-dimensional matrices cease proliferation [9, 10]. In this study we confirm that polymerized, but not monomeric, collagen matrix have a profound antimitotic effect on mesangial cells. Cell cycle analysis revealed that this growth suppression was associated with the G0/G1 arrest.

Growing evidence suggests that E2F transcription factors play a central role in the progression of the cell cycle. This study examined the expression of E2F-1 and E2F-4, the major members of the E2F family. Expression of E2F-1 was transcriptionally inhibited at the early phase of culture on polymerized collagen, whereas it was constantly expressed in mesangial cells on monomeric collagen. E2F-1 is reported to be a target for both growth-stimulatory and growth-inhibitory signals [33–35]. Our present results are in agreement with this view. Importantly, on polymerized collagen, down-regulation of E2F-1 preceded complete growth arrest and was already observed after 24 hours, when more than 20% of cells were in S-phase. On monomeric collagen, down-regulation of E2F-1 was not observed, even after 96 hours when the S-phase fraction decreased to less than 12%. These observations imply a possibility that repressed expression of E2F-1 is not simply the result of growth arrest.

The family of retinoblastoma susceptibility gene proteins, pRB, p107, and p130, play major roles in the suppression of cell cycle progression, mainly through interaction with E2F family proteins [16]. In mesangial cells cultured on polymerized collagen, p130 was quickly dephosphorylated. After 48 hours, most of the p130 was converted to the nonphosphorylated form. Similarly, pRB was also gradually dephosphorylated when the cells were cultured on polymerized collagen. Our data from

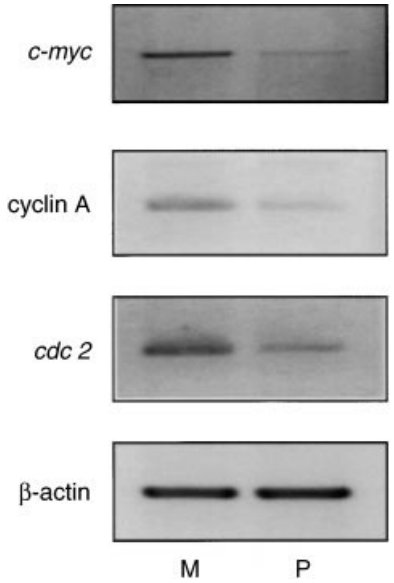


Fig. 5. Effect of polymerized collagen on mRNA expression of E2F-controlled genes. Total cellular RNA was isolated at 72 hours of culture on either monomeric (M) or polymerized (P) collagen and subjected to RT-PCR analysis for c-*myc* (25 cycle), cyclin A (25 cycle), and *cdc 2* (25 cycle) mRNA expression as described in the legend of Figure 2. The expression of β-actin gene (19 cycle) is shown as an internal control.

gel retardation assays further provided evidence that polymerized collagen matrix induced the accumulation of E2F-pRB and E2F-p130 complexes, which are capable of binding to E2F sites. Previous studies have shown that nonphosphorylated forms of RB family proteins, upon binding to E2F, switch E2F from a transcriptional activator to a repressor [21, 22, 30]. Thus, E2F-pRB and E2F-p130 complexes generated in the cells cultured on polymerized collagen may function as suppressors of the E2F-1 gene. Expression of another RB family protein, p107, was transcriptionally inhibited by polymerized collagen. It is consistent with a recent report showing that the expression of p107 is dependent on E2F [36]. Moreover, the antibody perturbation experiment showed that in contrast to anti-pRB and anti-p130 antibodies, the shifts in electromobility of E2F was not attenuated by the anti-p107 antibody. Theses data suggest that p107 is not integrated in the growth inhibition by polymerized collagen in mesangial cells.

Extracellular matrix is a key factor that controls the phenotype of mesangial cells. Previous investigations have shown that mesangial cells cultured within three-dimensional ECMs exhibited differentiated phenotypes, as well as attenuated mitogenic activity [6, 10, 37]. The contribution of RB family proteins to cell cycle arrest during the differentiation processes is cell type dependent. Corbeil, Whyte, and Branton detected E2F-pRB and E2F-p130 complexes during the induction of quiescence and differentiation of muscle cells and neuroneal cells [38]. Rampalli et al identified pRB and p107, but not p130, as primary regulators of E2F activity in lens fiber cell differentiation [39]. pRB is reported to play a crucial role in the differentiation as well as a negative regulator of the cell cycle [40–42]. Together with our present results, functional E2F-pRB and E2F-p130 complexes may be prerequisite for the induction of quiescent phenotypes of mesangial cells.

Cells anchor to ECM through cell surface receptors, integrins [3]. Wary et al reported that a protein Shc plays a role in activation of mitogen-activated protein (MAP) kinases and progression of cell cycle in response to integrin ligation [43]. A recent study by Miralem and Templeton demonstrated that a broad spectrum of signaling cascades, including focal adhesion kinase, MAP kinase, protein kinase C, and calmodulin-dependent protein kinase, are repressed in mesangial cells cultured on polymerized collagen [44]. At present, however, it is unknown how the engagement of integrins to polymerized collagen leads to suppression of the signaling pathways. Moreover, it is also unclear how these suppressed signals are involved in modulation of cell cycle machinery. Koyama et al reported that polymerized collagen could suppress p70 S 6 kinase, a potential regulator of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ in arterial smooth muscle cells [45]. Together with other previous reports [29, 46], $p27^{Kip1}$ may be responsible for dephosphorylation of RB family proteins and repression of E2F-controlled gene expression observed in this report.

It is known that aberrant regulation of cell cycle is related to certain pathologic conditions, including glomerular diseases. These disorders are often associated with up-regulation of matrix-degrading enzymes, that is, matrix metalloproteinases (MMPs), and destruction of surrounding ECMs [47]. In glomerulonephritis, an accelerated breakdown of ECMs and aberrant cell cycle regulation are concurrently observed [14, 15, 48]. Our current data further support the importance of three-dimensional ECM for controlling mesangial cell behavior and may provide the molecular basis regarding how structural alteration in surrounding ECM modulates cellular mitogenesis.

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